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JOANNE STUBBE: OK, so I think what I'm going to do is I'll give you an overview of what the hypothesis is that we'll be talking about in class the next time, probably the next time and a little bit into Wednesday. And then this module will be over. So this is covering the second lecture, the good NADPH, the good function of NADPH oxidases.

OK, so it's taken from this Kate Carroll's paper who-- and we'll continue where we left off last time. We'd gone through part of the paper you were supposed to read in Angewandte Chemie, but we hadn't gotten through the whole paper. And there were parts of it that I wanted to look at in more detail.

But this is sort of a model. So this is really sort of-- and I think the model is easy to understand. And these kinds of models have been in the literature for 10 or 15 years. But really, there hasn't been experiments that sort of show how this all goes together using multiple methods. And the underlying theme-- and let me also say, in the handout I will give you, I've given you an *Annual Reviews in Biochemistry* paper by the [INAUDIBLE] lab, which shows how much more complicated life is than this simple cartoon.

OK, so everything we look at, you start out with the simple cartoon. But when you study it, the more you study it, the more complicated it gets. So if you care about things only at the circle and square level, then you get a cartoon. But if you really care about how it works, you have to do a lot of work to figure it out.

OK, so this sort of model is similar to all-- we're going to be talking about epidermal growth factor. And it's similar to how many growth factors work that I think wasn't outlined in this paper, but maybe it was the first paper, that this model is sort of a generic model for many of these systems. Although, the signaling pathways are distinct.

So you have the epidermal growth factor receptor, which has a single transmembrane region. And this little-- what is this little domain in the cytosol? That's the tyrosine kinase activity. And it can react on itself.

And this little ball is the growth factor. And so the model has been that, when the growth factor is around, that the proteins can dimerize. And so that's what's indicated here. So this is the model that was taken out of the paper.

And in the presence of ATP, they can phosphorylate each other. OK, so that kinase domain has become active. And one of the questions is how do you know that phosphorylation makes it active. So you have to design experiments to test this.

And if you go back, and you think about in introductory biochemistry course, you saw many cascades. And sometimes, phosphorylation activates. And sometimes, it inactivates. So all of that needs to be studied in some fashion.

And so what we're going to be talking about in the module 7, which is the reactive oxygen species, is this protein, the NOX protein, NADPH oxidases. And what's the function of NADPH oxidases? What are the cofactors required? Anybody get that out of the paper?

So you have an NADPH oxidase. So obviously, what's one of the cofactors?

AUDIENCE: NADPH.

JOANNE STUBBE: NADPH. OK, so what is another cofactor? Did any of you look at that? Or you haven't read the handout, first handout on reactive oxygen species, because it's also-- there are six isozymes of these NADPH oxidases. And they all form different complexes, but the basic chemistry is the same in all of them.

So does anybody know? No. Any guesses? Ultimately, we have to convert the function of this protein-- hopefully, you know this is an unusual function. It converts oxygen into superoxide, which is one of those reactive oxygen species we saw this morning.

OK, and this is the only known protein that does that, that's specific function is to generate superoxide. A lot of times, you do generate superoxide, but it's an artifact of uncoupling reactions or other consequences inside the cell. So we have NADPH, and that's on this side and the cytosolic side. This is the extracellular side. No guesses as to what the cofactor is?

OK, so it has two kinds of cofactors-- FAD, OK, and the second cofactor are two heme b systems. So you have three cofactors-- FAD that are bound within the membrane and two hemes. And so that's true of all these systems.

So NADPH is a two-electron donor. And what are we generating over here? We're generating superoxide from oxygen. So that's one electron. So the major function of NADPH is not to go from two electrons to one electron, but it transfers electrons to the flavin. They have similar redox potentials inside the cell.

And then the flavin, because it's long and planar, can do both two-electron and one-electron chemistry. And if you have a heme, you can only do one-electron chemistry because you toggle between iron(III) and iron(II). So we'll talk about that in class next time.

But somehow, you do chemistry over here. And this to me is the most amazing thing, and it should have upset you when you read this paper. OK, you should now start-- we're at the end of this course. You should now start, you know, you say how the heck can that happen. I don't believe any of this. This is the way I read all papers. I don't believe of this. Right?

[LAUGHTER]

So I come in. I'm at the one extreme. I don't believe anything I read because I think people are really sloppy experimentally. But you guys should start thinking like that.

So we're going to generate superoxide. And it's on the outside of the cell. OK, This is a transmembrane system. So is there anything that links, if you read this paper carefully, and we'll look at data, but is there anything that links these two proteins in this paper that you were supposed to read?

Which, even if you read it, there's not that much data there. You know, I guess there is a lot of data within each panel. But what the data is in each panel, they show you something, and then they quantitate it adjacent to it. So it's the same data just transferred into some kind of a bar graph because that's the way biologists do things, rather than actually drawing some kind of-- showing you some kind of a model with kinetics on it.

So what do we know about these two proteins from what's in this paper? Any of you get anything out of that?

AUDIENCE: They form a complex.

JOANNE STUBBE: Yeah, they form a complex. So we'll come to that. But one of the experiments they did was they had antibodies to this, antibodies to this, and they showed you a picture where green and red went to yellow. And that was the evidence that these things might be linked.

So what's unusual is that you're generating superoxide extracellularly. OK, and it's charged. In general, it doesn't go through membranes. But what you see, if you read the paper again, is the function of the NOX protein is to modulate the activity of the tyrosine kinase, which is intracellular.

So the first thing that's bizarre is how do you get this superoxide inside the cell. OK, so that should have bothered-- it still bothers me. I mean, we have a model for how this works, but that's what the model is in all of these systems. And superoxide we'll see in the presence of protons.

And protons, you don't have to have very acidic to have protons. It can cause disproportionation of superoxide to form oxygen and hydrogen peroxide. That happens actually quite fast.

So the proposal is that somehow this disproportionates to form hydrogen peroxide. And it's the hydrogen peroxide that gets inside the cell. Hydrogen peroxide is neutral. Superoxide is charged.

OK, and so the model has been that this molecule, AQP-- does anybody know what that was? Did--

AUDIENCE: It's an aquaporin.

JOANNE STUBBE: Yeah, so aquaporin, so what does that do? They won the Nobel Prize for this two years ago for it supposedly is a water channel.

AUDIENCE: For water, yeah.

JOANNE STUBBE: Yeah, and we three-dimensional structures that people have gotten very excited about. So hydrogen peroxide sort of looks like water.

AUDIENCE: And you can fit it through the aquaporin pore.

JOANNE STUBBE: Yeah, so well, that's the model. Yeah, and there is a model from people that have looked at the structures, have modeled that this could go through. So now you generate hydrogen peroxide.

OK, and so the hydrogen peroxide, what we've been talking about is post-translational

modification by reacting-- what are we reacting? We're reacting a sulfhydrl group. Let's see if I brought my chalk. I don't have any good chalk. They don't make fat chalk anymore.

All right, so they're converting-- we what did we talk about last time? We were talking about this reaction. This was the major focus. So we're forming the sulfenic acid.

OK, and this whole paper is about, number one, do you form sulfenic acid inside the cell. And does it affect signaling? That's the focus. And her hypothesis was that it does.

And so then you need to know something about the signaling pathway, which other people have studied. And so there are apparently two signaling pathways, which we're not going to pay that much attention to, but you need to pay attention to it enough because some of the figures in the paper-- what did some of the figures in the paper look at? Anybody remember that? Were you confused by that? It was in figure 1 if I remember correctly.

AUDIENCE: Yeah, was it phosphorylation of these [INAUDIBLE].

JOANNE STUBBE: Right, so they looked at phosphorylation. And how can you do that? You can have an antibody.

AUDIENCE: Western.

JOANNE STUBBE: Yeah, by some kind of Western. So in the first figure, if you hadn't looked carefully at this figure, you wouldn't know why they were looking at phosphorylation at all. But what they're trying to look at, like in many of these systems, is a signaling pathway where something phosphorylates then dephosphorylates. OK, so these are the two signaling pathways, and they integrated that into their analysis.

And so now the key questions, if you look at the tyrosine kinase activity, why would you think-- what did you learn in this paper sort of grossly about the potential for sulfenylation? Anybody read? Did anybody look at the supplementary information? I think that's always the best part of the paper.

See, this is what the issue is nowadays. Now I'm digressing again. So I'm allowed to do this. I only have another five lectures to teach, and I'm finished anyhow. I mean, to me, it's sort of like that's where all the data is. And so nowadays, nobody puts data in papers. OK, it's impossible to evaluate a paper. It's all in the supplementary information.

And what irritates the hell out of me is that most people just dump it. That is they don't present it in a way that's thoughtful so that someone like me-- I don't care. Most people don't want to read all the details. That's fine. But then if you're going to present it, it should be so that I can read it and go back and forth and figure out what's going on.

Anyhow, here's a case where they talk-- do you remember that they talked about the kinase activity in this paper? Anybody remember that? We will get to that data if I get off this slide.

AUDIENCE: I think in Blake they talked about the fact that it enhances the kinase activity if you have the post-translational modification.

JOANNE STUBBE: Right, so what did they measure? Did you look at that? There's a figure where they focused on that. I can't remember what figure it was, probably figure 4, 5. So were you confused? It was one thing where you should have been confused because you wouldn't know what any of the words meant.

So well, no. So what did you do? I mean, I had to do the same thing. I went and googled it and looked up, and I figured out what the words meant. And then I understood. And we're going to go through that because the reagents are a key thing. You need to understand what the reagents do to be able to think about what you're looking at.

So anyhow, we'll come back to this, but, you know, we have-- this is a major target of drugs. There are drugs that are used clinically in treatment of cancer that target the epidermal growth factor receptor. And they inhibit the kinase domain, which were used to ask the question-- remember we talked about this last time, the importance of reversibility of this.

And if you modify it, what is the effect? OK, so is the effect that you alter the downstream signaling, whatever is downstream if you know something about that? Or the other effect can be is do you modulate the catalytic activity. And does anybody get that out of one of the figures? Or was this paper so hard we didn't even get that?

Look in figure-- I need a copy of the paper. Oh, I don't think this paper was that hard, but you had to work at it because the figures were small, and there was a lot of information. So if you look at figure 5, anybody look at figure 5? We'll come back to that in a minute.

So to me, when I look at-- and you'll see the way I write this down. You have a panel of stuff. You have a panel of all these figures. Well, you know, usually, the title tells you what the whole figure is about. But then at the end of this, you'd like to be able to look at the data without any

input from the person writing the paper and draw your own conclusions.

So at the end of this, this is what I do in every paper. I look at the figures, and then I draw a conclusion from the figures. And if I can't draw this-- I do this with my students too. If I can't understand from looking at the figures what the key conclusions are, they didn't write a good paper.

So this here, if you look at figure 5, you will see that there's activity. And so remember, if you're going to do this, why would you want to do it? It's got to have some effect. Right? Or it's not interesting.

And so the key issue is that you could get chemistry like this that's happening, and it isn't interesting. And it's very challenging to look at this chemistry inside the cell because thiols get oxidized fairly easily. So what you want to do is not only-- and this is what we were focused on last time is seeing if this happens. OK, we haven't gotten to inside the cell yet.

But then the question is is it connected to signaling. And that could be related to the activity of the tyrosine kinase, which triggers off the phosphorylation cascades. In a way you, you have to look up what other people have done to sort of understand that.

OK, so now we have the issue is that we made hydrogen peroxide. OK, supposedly, by this model, it's gotten inside the cell. And so now the model is that it does this. OK, so what you see is the issue with this model, which is why people have been fighting over this, is the rate constant for this reaction. Even if you have a thiolate, it's not very fast. OK, so that's something we'll talk about in class.

But the Winterbourn paper, when you read that, focuses on the rate constant. So this is a second order rate constant. It's like 1 per molar per second, really slow. So if this was happening over the period of hours, and your signaling is finished in 15 minutes, you've got a serious problem. So you have to deal with that problem. And people are finally dealing with that problem.

And there are proteins you'll see in the next lecture that, you know, the pK of the sulfur isn't all that perturbed, but they're able to react with hydrogen peroxide much, much faster. And that that's going to be a key piece of information. So if you get modification, then the question is what is the consequences of the modification, which is what the paper is about.

So the other key player in all of this, if you have something phosphorylated, so here we are something phosphorylated. So that's a kinase. It phosphorylates itself. But then whenever we have a kinase, we usually have something that clips off the phosphate, which is a phosphatase. So that's PTP.

And we'll see that the phosphatases we're dealing with all have cysteines in the active site. OK, and the cysteines are all-- so the PTPs all have an active site cysteine. And this active site cysteine-- you've seen this over and over again-- is involved in covalent catalysis. OK, we've seen hundreds of examples of this now. So you might not know what's involved, but that would be a good guess based on everything we've seen.

So it turns out the question is this is the active form. OK, so how could you shut it off? You might be able to shut it off by sulfenylation. So this would be active, and this would be inactive.

So what you're looking at is, again, another method of post-translational modification-- this is the hypothesis-- that can affect the activity in these cascades. And does anybody remember what conclusion people drew about the phosphatase? That was another figure in the paper.

Does anybody remember? There's a lot of information. But in the end, there aren't that many conclusions you could draw. But part of the problem is that I haven't gone over this cascade in lecture.

AUDIENCE: They were dark.

JOANNE STUBBE: So I realize that's putting you at a disadvantage. But you've had a couple of weeks to read this now, so yeah.

AUDIENCE: They were differentially sulfenylated depending on whether or not you had EGF present.

JOANNE STUBBE: Right, so they were differentially-- so nobody knew what the phosphatase was. OK, so they had a bunch of candidates because we have the whole genome sequence. We know what all the phosphatases are. Just like we know there are 500 kinases, there are 100 phosphatases. And they know which ones are connected to certain kinds of signaling pathways.

So one of the key conclusions from this paper is that they identified, or they claimed to identify-- you may or may not-- when we get that far, you can look at the data and see whether you believe that. Based on what they reported here, they claim to know that it was SHP. I think it is. I don't remember the name of the phosphatase, but that's the one that was modified. And it

turns out even the phosphatase can be modified further in a cascade by proteins called peroxiredoxins, which aren't in this paper.

Anyhow, so that's the overview. And what I wanted to do was spend a little bit of time going back through what we had gone through last time, not this part. We're going to go through this very fast because we got this far. Again, the development of a specific reaction with either iodo-dimedone or dimedone to modify either the sulfenic acid or the sulfhydryl group.

OK, and the issue is you can do this inside the cell. These are cell permeable. But then how do you ever find it? Right, so you've got 10,000 proteins. They can all get modified to some extent. We don't know how much of the protein is there. We don't know whether it's been partially modified. But there's no way to identify this currently.

And of course, the first thing is you're assuming that the linkage is stable. That's important. But even so, there's no handle on this. So the focus of the *Nature Chemical Biology* paper was to figure out how to make this so you could find this inside the cell and use this in some way. And so the mass spec method we had focused on last time, which is also used again in this paper, but if you didn't read supplementary information you won't know that, is they use isotopically labeled materials.

OK, so this is only if you have an extra mass of 6. You have the deuterated methyl groups. And that means you have a sulfenic acid that you've modified because she's shown that it's specific versus the methylated, which reacts with thiols only. So that's the basis of the assay.

And again, it's not easy to find a reagent that allows you to do this. And this is where we were last time at the end of the class. I didn't get this far with all the classes, and I can't remember who was in the class. But we were looking at this. This was just proof of concept.

So we have a glutathione peroxidase, which was also in the current paper. And what do we know about the glutathione peroxidase in this paper? If you read the paper, did this ring a bell from the previous paper? So what was unique about glutathione peroxidase?

AUDIENCE: It has an active site cysteine

JOANNE STUBBE: Yeah, it has an active site.

AUDIENCE: --that they can use to validate their approach.

JOANNE STUBBE: So it has an active site cysteine, which can get modified. There's something reactive about that. And it catalyzes. It has peroxidase activity. So it plays a very important role in controlling these reactive oxygen species. So it's a small protein that's been very well characterized.

And so if you look here, what do you see? This is where we were at the end of the lecture. What do you see? So this is an in vitro experiment, not an in vivo experiment. So we're in the test tube. And so what do you see?

So they treated it either here with dimedone, OK, which labels sulfenic acids, or they treated it with iodo-dimedone. And so what did that tell you? This is where we were last time.

When you looked at that, did that say anything to you without looking at the analysis out the other side? So you're looking at the figure. What did you think when you looked at the figure? What's your name?

AUDIENCE: Nicole.

JOANNE STUBBE: Nicole, what do you think?

AUDIENCE: You can see that, as hydrogen peroxide increases with the dimedone, the levels increase. But with the iod0-dimedone, the levels decrease.

JOANNE STUBBE: OK, so that's good. And then you can say one more thing. And what you could say is, in this lane-- so this is where they're looking at the iodo-dimedone, and there's no hydrogen peroxide. What does that tell you? So that's an extra piece of information that was more subtle out of this.

So if you look at this, you could think about it. You could do it. So look at this, so we're increasing hydrogen peroxide. And you saw it increases. Here we have just cysteine. Of the 100%, we're hitting it with something that reacts with sulfhydryl groups, the iodo. And look at this compared to this. What do you see? What does it look like?

AUDIENCE: It looks like there's more [INAUDIBLE].

JOANNE STUBBE: So that's exactly it. And it's the eyeball method. So you can't tell anything by the eyeball method. You have to have a way of quantity-- we talked about phosphorimaging. That's what people do.

But what this tells you is, if you looked again at the details, you know, they use 50 micromolar

of the protein. And they went to I think with 100 micromolar. And even when they went to 100 micromolar, they didn't inactivate. They didn't modify all of the GPx-3.

OK, now would you expect them to? I don't know. They probably tried a lot of different conditions. I mean it's concentration dependent. It's time dependent. And that wasn't given in the details.

OK, but this tells you then-- that takes you to the next one of the sets of data here, which you could have gotten in some form by looking at that data. So now what you're looking at is they're looking at a ratio of, you know, what's sulfenylated versus what's a sulfhydryl plus sulfenylated. And what do you see? Cysteine 36 is known to be at the active site, but they showed that in this experiment. And you see that you don't reach 100% labeling.

OK, so that you saw in the previous set of data. You couldn't tell what the ratio was. And this is sort of you're just looking at this is the mass. OK, and if you know what the protein is, remember, and you cleave the protein down with trypsin, you're going to get all these little peptides out. That's what we talked about last time.

And we're looking at charge-to-mass. We're looking for charge-to-mass differences in ratios depending on what the charge is for deuterium versus protons to tell whether it's sulfenylated, or it's just an SH group.

And so what they found was this charge-to-mass of 541 versus 554. And that told them-- if you look at the sequence that I told you in the computer can you analyze all this, it told you you were looking at peptide 36 to 43. And we know cysteine 36 is within that peptide. So that didn't tell us that the modification is based out that cysteine. But then you could go back in, and you could sequence. Yeah?

AUDIENCE: Why is the m/z different by three?

JOANNE STUBBE: Because you have charge. Because of the charge of the system.

AUDIENCE: [INAUDIBLE]

JOANNE STUBBE: Yeah, so you can get this at every-- you know, depending on how many masses you see, you can still pick up the data, but the number will be different. OK, so you would like to have six, but you might not be in that mass range. I mean here you would be. So you could have looked at that to have a difference of six.

So this tells you that you're not getting complete sulfenylation. So then the question is did any of you recognize that when you looked at the paper. Or did it just go by you? What could be going on?

So we already talked about the fact that maybe they didn't do the experiment right. They didn't have high enough concentrations. They didn't let it go long enough. Let's ignore that, OK, because they're good scientists. With some people, you might not want to ignore that.

But then the question is what could be accounting for this result.

AUDIENCE: Antibodies they used.

JOANNE STUBBE: The what?

AUDIENCE: They antibodies they used may not have the [INAUDIBLE]. So maybe we should [INAUDIBLE].

JOANNE STUBBE: OK, I mean, you know, you're going to use enough antibodies to be able-- you've go to check that out. So that would be another control you would need to do to make sure everything is under control. OK, so what we talked about last time in addition is hydrogen peroxide can oxidize this to these things as well. It's much slower.

And so you don't know how would you look for this. So one explanation is that you have some competing rates-- again, we don't know anything about it-- but that you had changed it and that the dimedone no longer reacts with these forms based on the mechanism we described. So that's a possibility. And in fact, if you read the supplementary information, how might you distinguish between this and this?

Say you thought that this was happening. I mean we're missing a lot of stuff. So there's a number of explanations. And that's just one of them. So what method are we using?

AUDIENCE: Mass spec.

JOANNE STUBBE: Yeah, how can you distinguish this from this?

AUDIENCE: Per mass.

JOANNE STUBBE: Yeah, by mass. And so if you look at that, you're going to have a different mass, right, on your little peptide species. You'll have the peptide species. And if you look at the supplementary data, they show mass in both of these states. OK, so that suggests that that might provide an

explanation for what's going on.

Maybe you could think of a lot of other explanations. Here I won't go through this. But they looked at mutants. And they wondered if all the cysteines could be modified, OK, because cysteines are reactive. Although, the ones in the active site often have lower pKa's.

So this all-- there are thiolates. So you might expect the reaction to be faster. But again, the reaction, even with the thiolate, with hydrogen peroxide, is slow. OK, so that's not the whole story.

And in fact, I think it was in the *Nature Chemical Biology* paper, there are some proteins they look at that all the cysteines can be modified. So this should start making you nervous in terms of how important sulfenylation is if it contributes a lot, if all of these things are heavily sulfenylated.

And in this experiment, they made a few mutants, and then they repeat the experiments. And they see that they don't see any modification. So that suggests that the cysteine 36 is the one. In the case of in the test tube, glutathione peroxidase is the one that's interesting and is being modified. So it is selective. It doesn't say anything about the biology because they're not looking at the biology of GPx-3.

And so then this is the method that they used. So once you identify that peptide, now you can have a second mass spectrometer and use a method to do collision-induced dissociation, which allows you to sequence the peptide. And now you ought to be able to-- it depends on the stability and the fragmentation patterns, but we know peptide bonds can fragment in a number of ways.

And people have figured this out over the years, namely Klaus Biemann figured this out that we have B and Y. And so one reports on the C-terminus of the peptide, the other on the N-terminus of the peptide. And those are the fragmentation patterns that are observed most frequently. And lots of times, one side, you don't see both with equal intensity. They fly differently. But that allows you then to sequence, and that's what they did in these papers.

And this just is an example of-- this isn't from glutathione peroxidase, but you can see you can now assign. If you wanted to go through this, you can walk through all of this. And you can see B2, B3, B4, et cetera, and you can see Y1, Y2, Y3. And the two of them should add up to give you your peptide fragment back. I mean once you get used to looking at these things and then

understand the mechanism of fragmentation, which is what Biemann worked out, you then have a picture of sequencing by mass spec.

And this is just another example that, again, this is not related to this particular problem. But you should see all these B fragments, all these Y fragments. And the two of them should sum to give you the total mass of whatever your peptide is.

OK, so they then looked at glyceraldehyde 3-phosphate dehydrogenase is another control in the last paper. That's an enzyme in the glycolysis pathway that has a cysteine in the active site that plays an essential role in catalysis. And they asked the question does it get sulfenylated. So it's implicated in all kinds of regulatory mechanisms as well.

And they did the same experiment. And what did they see here? So this is, again, the same ratio, deuterium over the sum of deuterium and protonated. And here they see one.

OK, so this one, they are able to titrate stoichiometrically on this active site. So again, it's a question. What that's telling you, which is an issue in the end, is, you know, they all have different reactivities is what-- yeah?

AUDIENCE: In the other slide, was there only one cysteine in the protein that they sent, like the fragment of the protein that they sent through the mass spec?

JOANNE STUBBE: No, so they actually-- whoops. Where is it? Yeah, so this one-- no, so they were looking at-- so they mutated this so they now had two cysteines, the one in the active site, and they had another cysteine, cysteine 64.

AUDIENCE: So was it still measured by-- you said it reacts initially with--

JOANNE STUBBE: Well, you react it with both dimedone-- you react it with hydrogen peroxide, and then you react it either with dimedone or with iodo-dimedone. And what they found is they saw no-- so they found fragments. OK, so the fragment that they were interested in, since that was the cysteine, is 64, OK, with 58 through 67. So that's the mass charge 639. So that's what they focused on.

OK, and so then they asked the question do you see D6. OK, D6 would be indicative of sulfenylation, and they saw no sulfenylation. So they can get modification. And they pulled out that cysteine, but they get no sulfenylation. Only the 36 was sulfenylated.

So they did a whole bunch of experiments like this. This is again just proof of concept, but I

think this data, just comparing the two proteins they looked at, shows you that you have different reactivities, which I think is part of the issues with these labeling methods that you're trying to use in cells. Yeah?

AUDIENCE: So in the method where they were just looking at the cysteine 36, they got 50% sulfenylation.

JOANNE STUBBE: So 50% sulfenylation, right.

AUDIENCE: Yeah, and there were no other cysteines in that fragment because--

JOANNE STUBBE: There were no other cysteines in that frame. So if you look at the sequence, you know, I mean this is a small protein. And so you know-- OK, so and now let's move into today or the precursor to today's paper. But this was the major focus.

So what they wanted to be able to do, they had proof of concept. And now the question is how do we show that this can-- can we do this inside the cell? OK, so what are the issues inside the cell? I've labeled some here. But have you guys done any thinking about this?

So we want this thing. What do we need of any reagent we're going to use? And what you see-- I think they've probably tried 20 or 30 reagents. I think these are the ones that came closest to working.

But if you look at this, there was also-- it probably was in supplementary information. I don't remember. There it is maybe. Let me-- does anybody remember looking at a figure where they address this issue?

Nobody remembers.

AUDIENCE: Which issue? Yeah, which issue are you referring to?

JOANNE STUBBE: Oh, the issue is whether you're getting sulfenylation inside the cell. Yeah, OK, and which reagent? So the first thing you want to ask are you getting sulfenylation. And which reagent works best?

AUDIENCE: It's too deep.

JOANNE STUBBE: OK, so it's definitely in here. I don't remember which one it is.

AUDIENCE: They talked about it in the supplementary [INAUDIBLE].

JOANNE STUBBE: Is it? OK, so let see if it's 2D. So I have this on a slide. I can go forward.

So the question is which one of these guys do you want to use.

AUDIENCE: Alkyne.

JOANNE STUBBE: OK, why?

AUDIENCE: In one of their preliminary experiments, it worked the best.

JOANNE STUBBE: OK, so this is the experiment. So what is this? This is the experiment. So somebody want to describe this experiment to me? So these are the kinds of questions they're asking in this paper. OK, so this is the same.

This is the same in vitro. That's what we just did with the GPx. That's what we just went through. And what conclusion? You know it's really clean. And so you're looking at an antibody to dimedone. OK, that's what they're using.

And so what conclusion can you draw from this with respect to these three systems, these three reagents?

AUDIENCE: That they're comparing basically if you label with the N3 on the guy that you're trying to look at or with the alkyne. And in vitro, they--

JOANNE STUBBE: Right, not only N3, but two different N3's-- you know, what is the nature of the linker? OK, that's something you need to pay attention to.

AUDIENCE: I think that's in the supplementary. This is just with one linker. Yeah, they didn't do this experiment with the--

JOANNE STUBBE: OK, so here they have DAZ2 and DYN. So here they have-- whoops. I don't know where DYN2 is.

AUDIENCE: So that's with the alkyne.

JOANNE STUBBE: OK, so that's in-- so they're the same linker?

AUDIENCE: Yeah.

JOANNE STUBBE: OK, because I don't remember that. So that's what they did here. And then they clicked it. OK, we were going to talk about that. We haven't gotten there. And what did they end up seeing in

this particular reaction?

AUDIENCE: [INAUDIBLE]. Yeah, like they really that equal in vitro.

JOANNE STUBBE: So you see it here, hydrogen peroxide. So they sulfenylated. OK, so without sulfenylation, without hydrogen peroxide, they don't see anything. So that's good.

And then here, they have hydrogen peroxide. So they sulfenylated. This is with--

AUDIENCE: Alkyne.

JOANNE STUBBE: --the alkyne. And this is with--

AUDIENCE: Azide.

JOANNE STUBBE: --the azide. So both of these is seeing something. OK, so now what they do over here is do the same experiment inside these cells. OK, and so inside the cells, what are you going to end up seeing when you look at this?

AUDIENCE: More stuff that gets sulfenylated.

JOANNE STUBBE: A mess, yeah. So you see a mess. But what do you see here? So again, they're doing the same kind of thing. We haven't talked about the reaction yet of how you pull these out. But which one is most heavily modified? This one, and this is the one where you're using--

AUDIENCE: Where you label with alkyne--

JOANNE STUBBE: --alkyne and click with--

AUDIENCE: --and azide.

JOANNE STUBBE: --azide. So this is the one that-- is this the one they use? This is the one they should have used.

AUDIENCE: [INAUDIBLE]

JOANNE STUBBE: Yeah, so if you go back-- whoops. So if you go back over here, we're trying to decide which one we want to use. And so that's the kind of experiment they did to try to design. So what are you concerned about in this experiment?

The first thing is what has to happen with this molecule. I needs to get in the cell. OK, so I

guess they know it gets into the cell, but they didn't really do any experiments to determine how much got into the cell. And it could be one that could have gotten into the cell much more than the other, which would have given you the same result. Presumably, they looked at that. So it is cell permeable.

The key thing, I think, is in the cellular milieu, you've got to be able to do all these reactions. OK, but what is the issue in the cellular milieu doing these reactions? How do you decide how to do the experiment? What do you have to worry about?

AUDIENCE: You want to have-- I mean both of these are the case. You want to have some sort of chemistry that's orthogonal to all the processes in the cells.

JOANNE STUBBE: So we want to do that. So that's important. But OK, so that's one thing you have to worry about. We think that they have that under control. What else do you need to worry about?

AUDIENCE: That this doesn't alter the sulfenylation profile in any way.

JOANNE STUBBE: Well, I mean you have to be able to sulfenylate first. OK, what governs sulfenylation if you look at these, if you look at these molecules? So you sulfenylate. Let's assume you can sulfenylate. We can't control that if you've got hydrogen peroxide in there, if you've generated hydrogen peroxide. So they've somehow got to do that. And you sulfenylate.

Or maybe they don't. They do EGF stimulated reaction. Then what do you have to worry about with respect to these analogs?

AUDIENCE: How well those react with the sulfenyl group.

JOANNE STUBBE: Right, and so what do you have to think about?

AUDIENCE: What the time scale is with the sulfenylation equilibrium versus like this reaction reacting with that species.

JOANNE STUBBE: And so that's all bimolecular. And the rate-- and it's bimolecular meaning something might be there in nanomolar. Something might be there in micromolar. The rate of the reaction is automatically a factor of 1,000 fold difference. So how long do you let this go?

If you let it sit for a very long period of time, sulfenylation, we're talking about the importance of reversibility. So are there redox systems? There are that can remove the sulfenyl group. So designing the experiment to be informative is, in my opinion, not trivial. So looking at the

details is key. And you need to know a lot about the system, which I don't know anything about.

And then, you know, this has going to be able to get into the active site. OK, so the active sites have got to be big enough. We know it can do that in glutathione peroxidase and GAP dehydrogenase. We looked at that. But this needs to be long enough so, wherever the active site is, you can do some chemistry out here. So all of these things are issues that you have to deal with.

And then the thing we we were facing before, if we only labeled with this, we have no way to tell where the label is. And so again, the idea is that you use these things to click it to something that's going to inform you where the label is. OK, yeah?

AUDIENCE: Was it synthetically driven that the [INAUDIBLE] linker they install at the like [INAUDIBLE] position of the reactive site versus--

JOANNE STUBBE: My guess is yes, but I didn't read the papers carefully enough to know that. So in the paper that you were assigned, they had a lot of synthetic chemistry. So if you read the details, which is key, I'm sure a lot of these things are going to be driven by what's easiest to make. You need to be able to make large amounts of it. And these are now all commercially available. OK, so this is-- so yeah, I think it is. But to me, you would do an experiment--

AUDIENCE: To show that?

JOANNE STUBBE: --at different positions because one of them might be much, much more efficient at much lower concentrations. And you might be much better off. I don't know. Or you certainly might want to use more than one reagent because of the issues of trying to get this thing to react.

And then you have this question of, even if you get these things, how do they react. And most of you have probably heard about click chemistry since it was invented by-- it wasn't invented by Barry, but Barry popularized it. And it's copper-catalyzed in most reactions. But to do that, you do it in cell extracts, OK, because copper is really toxic to cells. So it's not useful for looking at this inside the cell.

And so the Bertozzi lab made a strained alkyne with a fluorine on it-- actually, Jeremy did that who was an Alice Ting undergraduate here-- that makes it click. But it's still not good in my opinion. This still needs a lot of work.

And so I think the best methods actually are the new methods that are coming out of this guy's lab where he makes these tetrazine analogs. And then he's made cyclopropenes. And they react much, much faster under mild conditions. And people are using these to put on fluorescent probes.

So anybody who is interested in that, you can read about-- this guy, he's done a lot of creative science. He's a young guy about Brad's age. But I read all his papers because I think there so interesting. There aren't many young people that I do get to. Anyhow, this guy is good.

OK, so the issue now is how did how does this happen. And how do you do the analysis? And so now that we're finished, none of you have come to the board yet. OK, all right, so the reagents used, you need to think about the reagents used. So I'm going back to the model. I already gave you the model.

All right, so one of the reagents they used is this guy-- dihydrochlorofluoroescein. What did they use that for?

AUDIENCE: [INAUDIBLE]

JOANNE STUBBE: For what?

AUDIENCE: For hydrogen peroxide detection.

JOANNE STUBBE: OK, but is it hydrogen peroxide? Do you know how it works?

AUDIENCE: No.

JOANNE STUBBE: So to me, the first thing you should do is you should have googled it. And so it's dihydro. So this is in the reduced state. I mean, I could draw this structure up on the blackboard for you, but the importance is that it's in the reduced state.

And furthermore, what does the DA stand for? So this is where the reagent-- you know, to me, I didn't have a clue either because I don't work on fluorescent probes inside the cell. So I immediately went and looked it up because I didn't know what it was, and I didn't know what I was looking at. And so here was one whole figure with this reagent. And so how could you understand what the reagent was telling you if you didn't know what it was?

And so what it's got-- I can't remember the whole structure. You can google it. But it's got a couple of hydroxyl groups. And the hydroxyl groups are acetylated. Why might they want to

acetylate? Why would they use the diacetate?

AUDIENCE: It's not fluorescent, right?

JOANNE STUBBE: It's not. The reduced form is not fluorescent.

AUDIENCE: So in order for it to-- well, in order for it to be fluorescent, you're going to have to take off the acetates, which will happen in the cell [INAUDIBLE].

JOANNE STUBBE: Right, so the first thing happens. Number one, but how do you get the reagent into the cell? It turns out so it depends on the pKa of this hydroxyl. And so the acetate groups facilitate uptake into the cell. Then it gets inside the cell, and the acetate groups get hydrolyzed off. It's in the reduced state. And so this molecule in the reduced state--

AUDIENCE: Is fluorescent.

JOANNE STUBBE: --reacts with, quote, "reactive oxygen species," unquote, to get into the oxidized state. And that's what becomes fluorescent. So it starts out in the reduced state. And it has two acetate groups, sorry, two acetate groups. OK, and so this gets into the cell.

And then you have-- you still have the reduced state. And now you have a hydroxyl. And now this with reactive oxygen species-- so this is still non-fluorescent. And we're going to talk about fluorescence next time. And then it gets in. It gets oxidized by reactive oxygen species and becomes fluorescent.

So that's the assay, but it doesn't just react with hydrogen peroxide. It can react with a lot of molecules. And in fact, the Collins paper that I talked about in class where I made some snide comment on it-- anyhow, I mean the problem was, in the original paper, people just used these things blindly thinking they're reacting with specific molecules. And in the last five years, there's been a huge number of people that have focused on making sensors specific for each reactive species. And that's really what you need to do if you're going to make a sweeping generalization about something like this.

So yeah, we're at the end of our time. But what you should do is go back, and there are a whole bunch of reagents in this paper that, if you didn't know what they were, there's no way you can understand the data. OK, so the first thing you did-- or when I looked at this, the first thing I did is I made a list of these reagents because I understand what was going on.

You know, like I never heard of-- what is it-- apocynin. That's a specific inhibitor of NOX2 isozymes. So there are a bunch of different isozymes. People are really interested in these therapeutically. So people have developed specific inhibitors.

If you look at these guys, which they call them the wrong thing. They have a longer name, but these are covalent and irreversible inhibitors of epidermal-derived growth factor receptor. OK, so you need to know that to be able to look at each one of these panels to figure out what the data tells you.

OK, so now I would suggest you go-- you might see this again. Maybe you'll see this on the final exam, some of this data. Anyhow, you should go back, and you should look at the data. This is what we've been trying to get you to do over this course is, you know, it takes a lot of energy to read a paper. that's one of the take-home messages from the course. OK, so we have finished.